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# Optimization of the Self-Assembly Method for the Production of Psoriatic Skin Substitutes

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Additional information is available at the end of the chapter

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## Abstract

Tissue engineering of the skin is used for various applications. However, to develop treatments for skin pathologies such as psoriasis, robust pathological skin models are needed. The purpose of the work presented in this chapter was to optimize the production of more reproducible psoriatic skin substitutes by modifying the original self-assembly method. Substitutes were produced according to the self-assembly method partially modified. The culture flasks of 25 cm<sup>2</sup> were replaced by 6-well and 12-well plates. Fibroblasts were cultured in 6-well and 12-well plates with ascorbic acid until they form manipulable sheets, which were superimposed and incubated for 7 days to form a dermal layer. Afterwards, keratinocytes were seeded on the dermal layer forming an epidermal layer. Then, the substitutes were raised to the air-liquid interface and cultured 21 days before being analyzed. Analyses demonstrated that psoriatic substitutes have a significantly thicker epidermis than healthy substitutes and the persistence of nuclear structures in corneocytes, with original and both modified methods. Immunofluorescence markers such as filaggrin, loricrin, and keratin 14 have confirmed these results. However, some differences were observed in substitutes produced with 12-well plates. Modifications made to the original method for the production of psoriatic substitutes are effective and lead to highly reproducible substitutes more suitable for pharmacological testing.

**Keywords:** tissue engineering, cell culture, skin substitutes, psoriasis, self-assembly approach, *in vitro*

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## 1. Introduction

### 1.1. Skin

The integumentary system is the largest and heaviest organ of the body [1, 2]. This organ is divided into three distinct layers: the epidermis (superficial layer), the dermis (intermediate layer), and the hypodermis (deepest layer). Its main function is to protect the body from external aggressions, such as chemical, mechanical, thermal, microbial, and UV rays [3, 4]. It is therefore a physical, biological, and immunological barrier. The epidermis, the outer layer of the skin, predominantly ensures this barrier function by a constant renewal of keratinocytes, the epidermal cells. Keratinocytes differentiated into five layers: from the *stratum basale* (*stratum germinativum*), in which skin stem cells are found, to the *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum*, which is the outer layer where keratinocytes have lost their nuclei and are completely keratinized [5]. Keratinocytes from this layer, also called corneocytes, will gradually detach to cause the phenomenon called desquamation. The highly regulated process of differentiation involves specific proteins to maintain this epidermal structure, and deregulation of these proteins expression can induce skin pathology such as psoriasis.

### 1.2. Psoriasis

Psoriasis is an erythematous-squamous dermatosis touching both men and women. This chronic skin pathology affects 2–3% of the world's population [6, 7], which correspond to approximately 125 M people [8]. This pathology is characterized by a hyperproliferation and an abnormal differentiation of keratinocytes resulting in reddish and whitish plaques [5]. At a cellular level, histopathological characteristics consist of acanthosis, parakeratosis, hyperkeratosis, agranulose, and papillomatosis [9, 10]. The disease's etiology is still unknown. However, environmental and immune factors, as well as genetic predispositions, would act together to trigger psoriasis [10, 11]. This disease seriously affects the quality of life of patients due to the appearance of their skin and the side effects of drugs. Existing treatments cause many severe side effects such as nephrotoxicity, hepatotoxicity, immunosuppression, teratogenicity, and no curable treatments have been found [12–14]. Moreover, several comorbidities may be related to psoriasis, such as major cardiac events, type 2 diabetes, and psoriatic arthritis [6].

### 1.3. *In vivo* and *in vitro* psoriatic skin models

The skin is a complex organ. Thus, the production of representative and reproducible skin models is a constant challenge. The use of *ex vivo* human skin biopsies would be more convenient, since with skin biopsies, it is possible to observe the mechanisms and interactions of the human skin. However, because of skin donor availability and inter-individual variability, the use of *ex vivo* biopsies is not practical, and thus, the development of new models is important. Over the years, there has been a lot of progress in the field of tissue engineering [15]. Tissue engineering of the skin is used for various clinical applications and in fundamental research such as for drug development. Now, with the development and optimization of *in vivo* and *in vitro* models, it is possible to research new treatments for a skin disease by studying, for

example the antioxidant and antiproliferative potentials, and the toxicology of molecules or extracts [16, 17], to study the mechanism of action of compounds [18] and to perform percutaneous absorption studies, and thus study the permeability of the skin, the diffusion rate, and the site of action of compounds [19, 20].

### 1.3.1. *In vivo* models

Many approaches are used to obtain animal models as representative as possible to the human pathology. Spontaneous mutations, like the homozygous asebia, xenotransplantation, like the severe combined immunodeficient mice (SCID) and the athymic nude mouse, and genetic models, such as the CD18 hypomorphic mice model, the K14/TGF- $\alpha$ , and the involucrin/INF- $\gamma$ , have been used over the years to study psoriasis but all of them displayed some limitations [21–23]. Animal models are mostly used to study specific aspects of the pathology. The development of a representative animal model can be expensive.

### 1.3.2. *In vitro* models

There are two types of models: monolayer models (dermal or epidermal) and bilayer substitutes. Monolayer models use only one cell type, keratinocytes or fibroblasts, and will be used to study a specific characteristic or to understand the role of a certain cell type in pathologies such as psoriasis. However, these models exclude interactions between different cell types. Bilayer models displayed two layers of skin: dermis and epidermis, which allow the study of skin complexity more representatively. The challenge of skin engineering is to reproduce the complexity and the functionalities of a pathological skin. There are different *in vitro* psoriatic skin models, which include interesting pathological features. Various pathological bilayer skin models were developed using a collagen gel as dermal equivalent. Most of these studies involve pathological keratinocytes seeded on a dermis made of collagen and fibroblasts [24, 25], but there are also studies where a full-thickness psoriatic skin biopsy is incorporated into the dermal equivalent [26]. These models have been useful to better understand the disease and the interactions between fibroblasts and keratinocytes [26, 27]. However, the main disadvantage of these models is the use of an exogenous material, which does not represent exactly the properties of the human dermis. To counter the use of exogenous material such as collagen, some research teams have used de-epidermized dermis to produce their psoriatic skin models [28–30]. Although these equivalents demonstrate several psoriatic features, the use of these models for pharmaceutical studies would require an excessive amount of skin biopsies. Thus, a pathological model free of exogenous material that can generate many samples at a time is still required for pharmaceutical research.

Our team has developed a psoriatic skin model based on a self-assembly method, which is free of exogenous material [31]. This model has been characterized towards its permeability, lipid organization and response to antipsoriatic drugs [32, 33]. This basic model has also been improved by the addition of other cell types such as endothelial cells in order to reproduce the angiogenesis observed *in vivo* [34]. These studies have confirmed that our psoriatic skin substitute model produced according to the self-assembly approach maintained many characteristics of the disease including the presence of a disorganized and thicker epidermis compared with normal skin substitutes [31]. This self-assembly approach allows the understanding of

pathological skin complexity through the possibility of: (1) dissecting step by step the mechanisms of skin pathologies according to which kinds of cells are present in the model at that time and/or (2) using various cell combinations such as healthy fibroblasts and healthy keratinocytes, which can be compared with healthy fibroblasts and pathological keratinocytes. Although the self-assembly method is very effective for the reconstruction of substitutes used in basic mechanisms studies, it required an optimization of its original protocol to consider a productive capacity of it in the pharmaceutical industry. Thus, the aim of this work was to improve the original self-assembly method to allow the reconstruction of more reproducible psoriatic skin substitutes that could be used for pharmacological testing.

2. Modified self-assembly methodology

As mentioned previously, our team has developed a model of *in vitro* psoriatic skin substitutes using the self-assembly method. In this present research, the production of the tissue-engineered psoriatic skin substitutes was done according to the self-assembly method partially modified, using 6-well plates and 12-well plates [31, 35] (Table 1 and Figure 1). All methods were also compared to the reconstruction of healthy skin substitutes. Briefly, pathological fibroblasts were cultured 28 days with Dulbecco-Vogt modification of Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 UI/ml penicillin G, 25 µg/ml gentamicin, and 50 µg/ml ascorbic acid until they form manipulable sheets. Then, these fibroblast sheets were detached, and two of them were superimposed to form a new dermal equivalent. Subsequently, they were incubated for 7 days to allow the fusion of the two sheets and thus form the new layer. After this period, pathological keratinocytes were seeded on the dermal equivalent to form a new epidermal layer. Seven days later, the substitutes were raised to the air-liquid interface to promote cell differentiation and obtain the different epidermal layers. Skin substitute biopsies were taken at 21 days after being raised to the air-liquid interface and analyzed by histology and immunohistochemistry.

2.1. Results

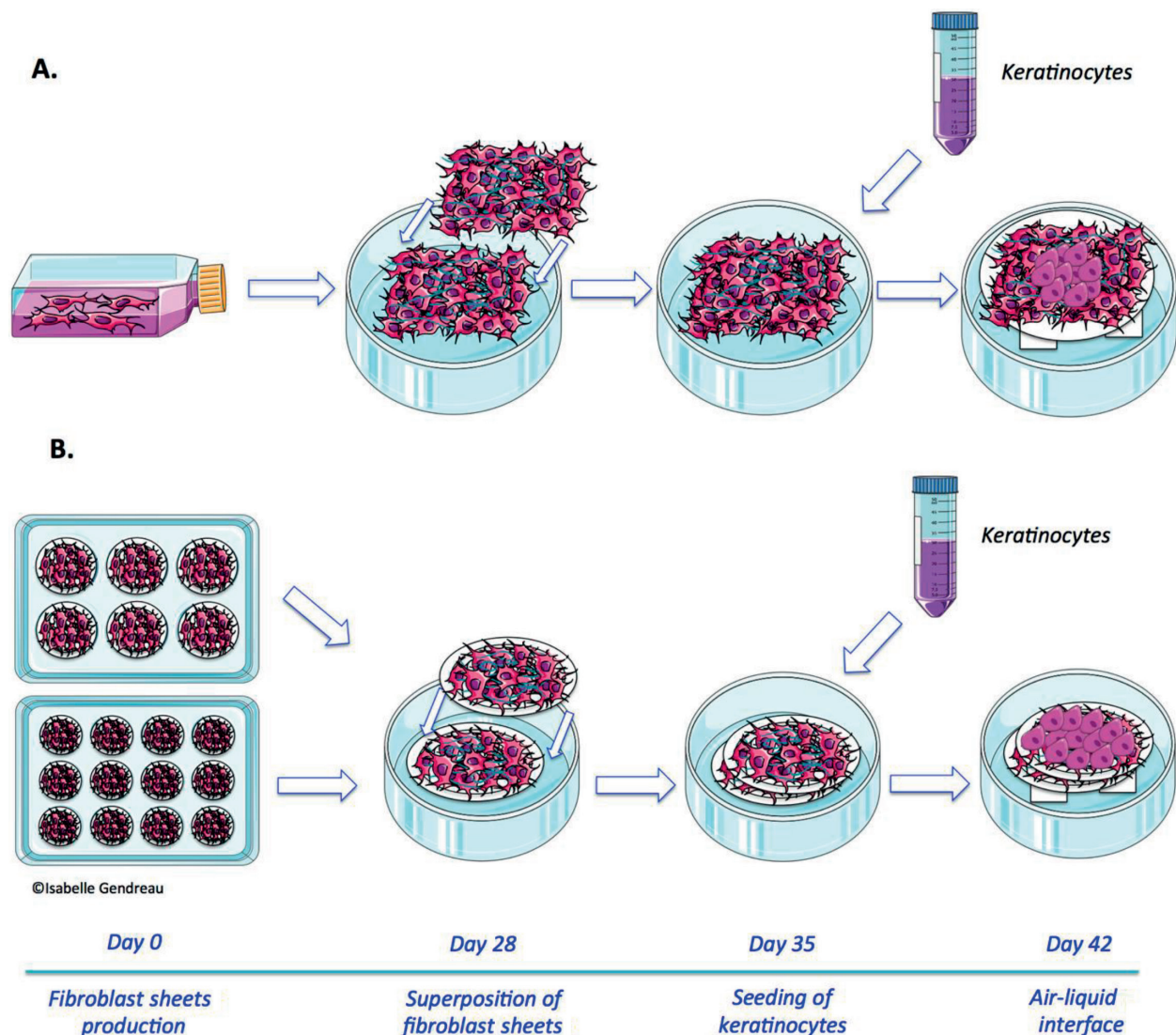
2.1.1. Macroscopic results

Healthy skin substitutes reconstructed using either the original or modified methods (Figure 2A–C) showed a uniform and opaque epidermis recovering all the seeding area within the anchoring paper (white contours). For substitutes produced with psoriatic cells,

Self-assembly method	Culture surface area (cm²)	Final size of substitute (usable) (cm²)	Volume of medium by fibroblast sheet (ml)	Anchoring papers
Original	25	3.87	5	Day 42
6-well plates	9.6	3.87	2	Day 0
12-well plates	3.8	1.27	2	Day 0

Table 1. Technical characteristics of the original method, the 6-well plate and the 12-well plate modifications.





**Figure 1.** Schematic representation of the self-assembly method. (A) Original self-assembly method: fibroblasts were cultured 28 days in 25 cm<sup>2</sup> flasks for the production of fibroblast sheets. At day 28, flasks were opened with a soldering iron, and two sheets were superimposed to form a dermal equivalent. After one week, a metal ring was deposited on substitutes, and keratinocytes were seeded into the ring. Then, substitutes were cultured one more week in submerged conditions. At day 42, substitutes were placed on an anchoring paper and were raised to the air-liquid interface for three weeks. (B) Modified self-assembly method: fibroblasts were seeded in 6-well or 12-well plates in which anchoring paper have been previously placed. After 28 days of culture, two fibroblast sheets were easily superimposed and solidified with Ligaclip®. Dermal equivalents were cultured seven days before keratinocytes seeding. At day 42, substitutes were raised to the air-liquid interface and cultured for three weeks.

those reconstructed according to the original method and the 6-well plate modifications (**Figure 2D–E**) showed an irregular and contracted epidermis. Psoriatic skin substitutes produced using the 12-well plate modification (**Figure 2F**) demonstrated a more uniform epidermis compared to other methods.

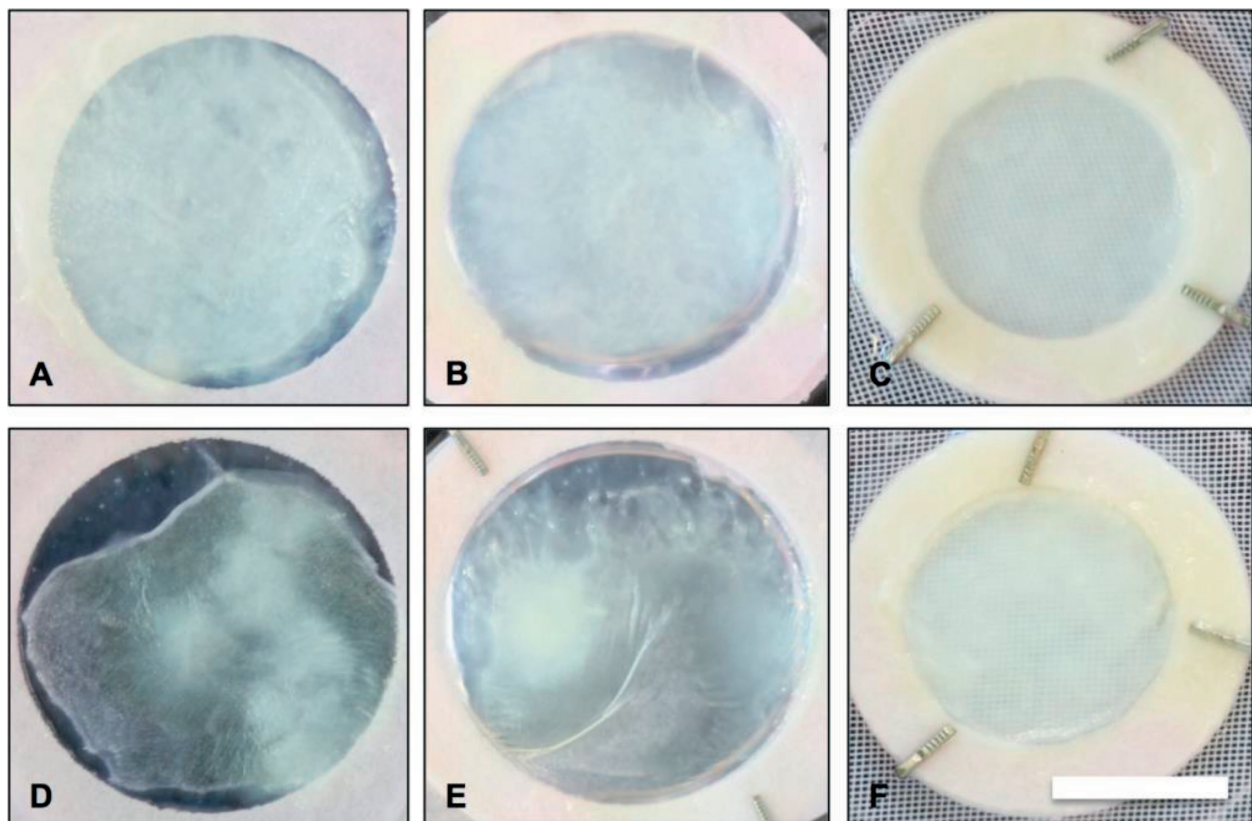
### 2.1.2. Histology

Healthy skin substitutes reconstructed according to the original method, and the 6-well plate modification (**Figure 3A and B**) demonstrated a well-differentiated epidermis and similar

characteristics. A thickening of the living epidermis was observed in substitutes produced with psoriatic cells using the original method, as well as in those reconstructed according to the modified protocols (**Figure 3D–F**). A less differentiated epidermis was observed in psoriatic substitutes produced according to the original method and the 6-well plate modification (**Figure 3D–E**), compared with the substitutes produced according to the 12-well plate modification (**Figure 3F**). The substitutes reconstructed according to the 12-well plate modification (**Figure 3C and F**) demonstrated a greater cell differentiation which results in a thickening of the *stratum corneum*.

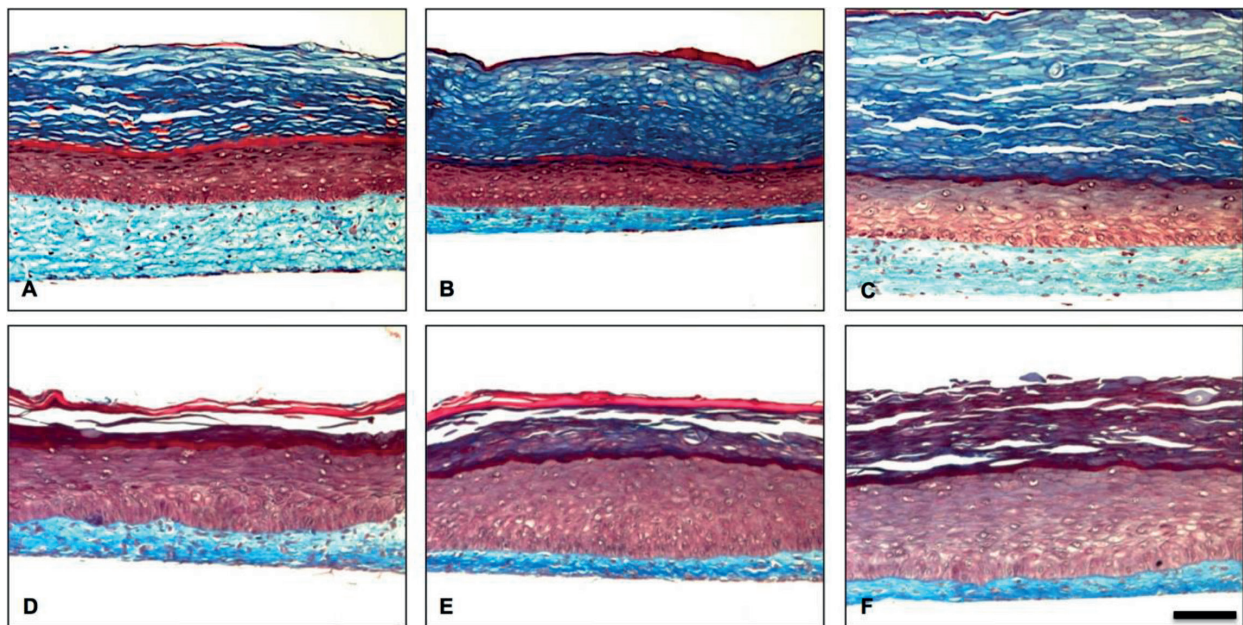
### 2.1.3. Epidermal thickness

Thickness of skin substitutes' living epidermis was measured with the AxioVision software. No significant differences were observed between the skin substitutes reconstructed with healthy cells according to the original method or the two new modifications ( $65.6 \pm 13.9$  vs.  $69.0 \pm 6.8$  vs.  $78.39 \pm 18.69$   $\mu\text{m}$ ; **Figure 4A**). Measurements of psoriatic substitutes thickness reconstructed according to the original method, and the 6-well plate modification did not show any significant differences between these two methods ( $93.50 \pm 18.9$   $\mu\text{m}$  vs.  $106.7 \pm 23.7$   $\mu\text{m}$ ; **Figure 4B**), while psoriatic substitutes produced with the 12-well plate modification demonstrated a significant difference compared with the original method ( $130.8 \pm 18.8$  vs.  $93.50 \pm 18.9$   $\mu\text{m}$ ).

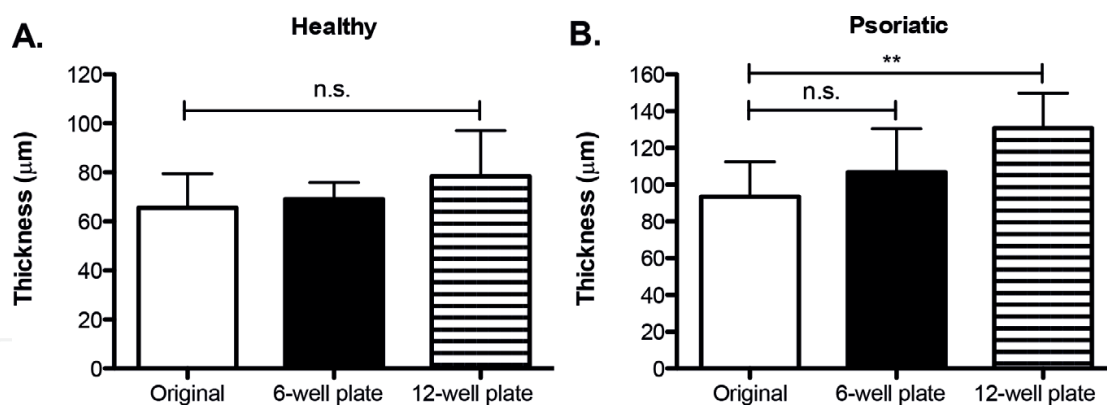


**Figure 2.** Macroscopic analyses. Macroscopic appearance of healthy substitutes (A–C) and psoriatic substitutes (D–F). Substitutes were produced according to the original method (A and D), 6-well plate modification (B and E), and 12-well plate modification (C and F). Three substitutes of each condition were analyzed, and the results were confirmed with three independent experiments. Cells from two different healthy patients and three different psoriatic patients were used (scale bar = 1 cm).





**Figure 3.** Histological analyses. Masson's trichrome staining of healthy substitutes (A–C), psoriatic substitutes (D–F) and substitutes produced with the original method (A and D), 6-well plate modification (B and E), and 12-well plate modification (C and F). Three substitutes of each condition were analyzed, and the results were confirmed with three independent experiments. Cells from two different healthy patients and three different psoriatic patients were used (scale bar = 100  $\mu\text{m}$ ).



**Figure 4.** Thickness of the living part of epidermis. (A) Measurements of healthy substitutes produced with the original method, the 6-well plate modification, and the 12-well plate modification. (B) Measurements of psoriatic substitutes produced according to the original method, the 6-well plate modification, and the 12-well plate modification. The statistical significance was determined using ANOVA test ( $p < 0.05$ ,  $n = 3$ ,  $N = 3$ , 90 measurements by condition). Data presented are means  $\pm$  S.D. (\*\*) =  $p$ -value  $< 0.001$ .

#### 2.1.4. Immunofluorescence analyses

Immunofluorescent markers were used to compare protein expression. Late differentiation markers, such as filaggrin and loricrin, were observed in order to compare the different skin substitute models. The *stratum granulosum* of *in vivo* skin is characterized by the presence of keratohyalin granules and the expression of late differentiation markers, since their synthesis depends on keratohyalin granules [36, 37]. Moreover, late differentiation markers are found

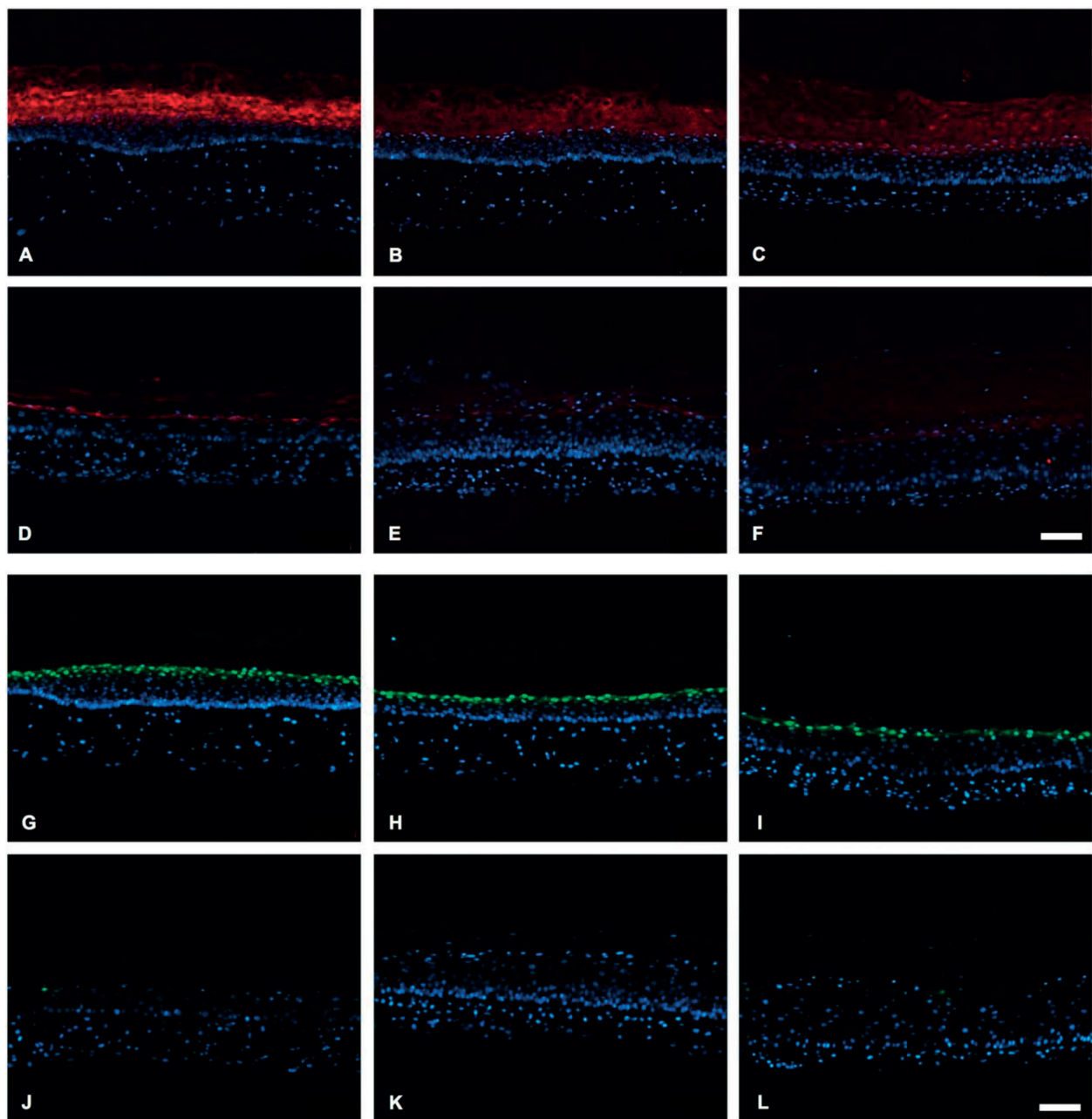


in the *stratum granulosum*. In healthy substitutes, filaggrin was expressed from the last layers of the *stratum granulosum* to the first ones of the *stratum corneum* (Figure 5A–C). In healthy substitutes produced according to the 6-well plate and 12-well plate modifications, a slight decrease of filaggrin was observed (Figure 5B and C). However, since keratohyalin granules are in lesser amount in psoriatic skin than normal skin due to abnormal keratinocytes differentiation [38], expression of late differentiation markers are downregulated. In our psoriatic substitutes, whether the original method was used or the two modifications to protocol were followed, filaggrin is reduced and almost missing (Figure 5D–F). Loricrin, another late differentiation marker found in *stratum granulosum*, was observed in healthy substitutes (Figure 5G–I). In the psoriatic substitutes, a decrease or an absence of loricrin was observed which is similar to *in vivo* psoriatic skin (Figure 5J–L).

Other markers, such as keratin 14, keratin 1, and laminin, were also observed. Keratin 14 (K14) is expressed in the basal layer of the epidermis and is gradually reduced until keratins 1 (K1) and 10 (K10) are synthesized. In healthy substitutes produced according to the original method and the 6-well plate modification, keratin 14 was normally expressed (Figure 6A and B), whereas in healthy substitutes produced according to the 12-well plate modification, this keratin was still present in the *stratum corneum* (Figure 6C). In psoriatic skin, this protein is expressed in all epidermis such as observed regardless of the method used in the production of psoriatic substitutes (Figure 6D–F). Keratin 1 staining showed no difference between healthy substitutes reconstructed according to the original method or the 6-well plate modification (Figure 6G–H), while this keratin was overexpressed in *stratum corneum* of healthy substitutes produced according to the 12-well plate modification (Figure 6I). In psoriasis, keratin 1 is decreased such as observed in the psoriatic substitutes produced according to the original method or the 6-well plate modification (Figure 6J–K), whereas psoriatic substitutes produced according to the 12-well plate modification showed a higher expression of this keratin in the *stratum corneum* compared to the other two (Figure 6L). Laminin expression was similar for all reconstructed healthy substitutes (Figure 7A–C), while for psoriatic substitutes, laminin expression seems more distributed through the dermis using the original method and the 6-well plate modification (Figure 7D–E). Laminin staining of psoriatic substitutes produced according to the 12-well plate modification (Figure 7F) was similar to the healthy substitute expression.

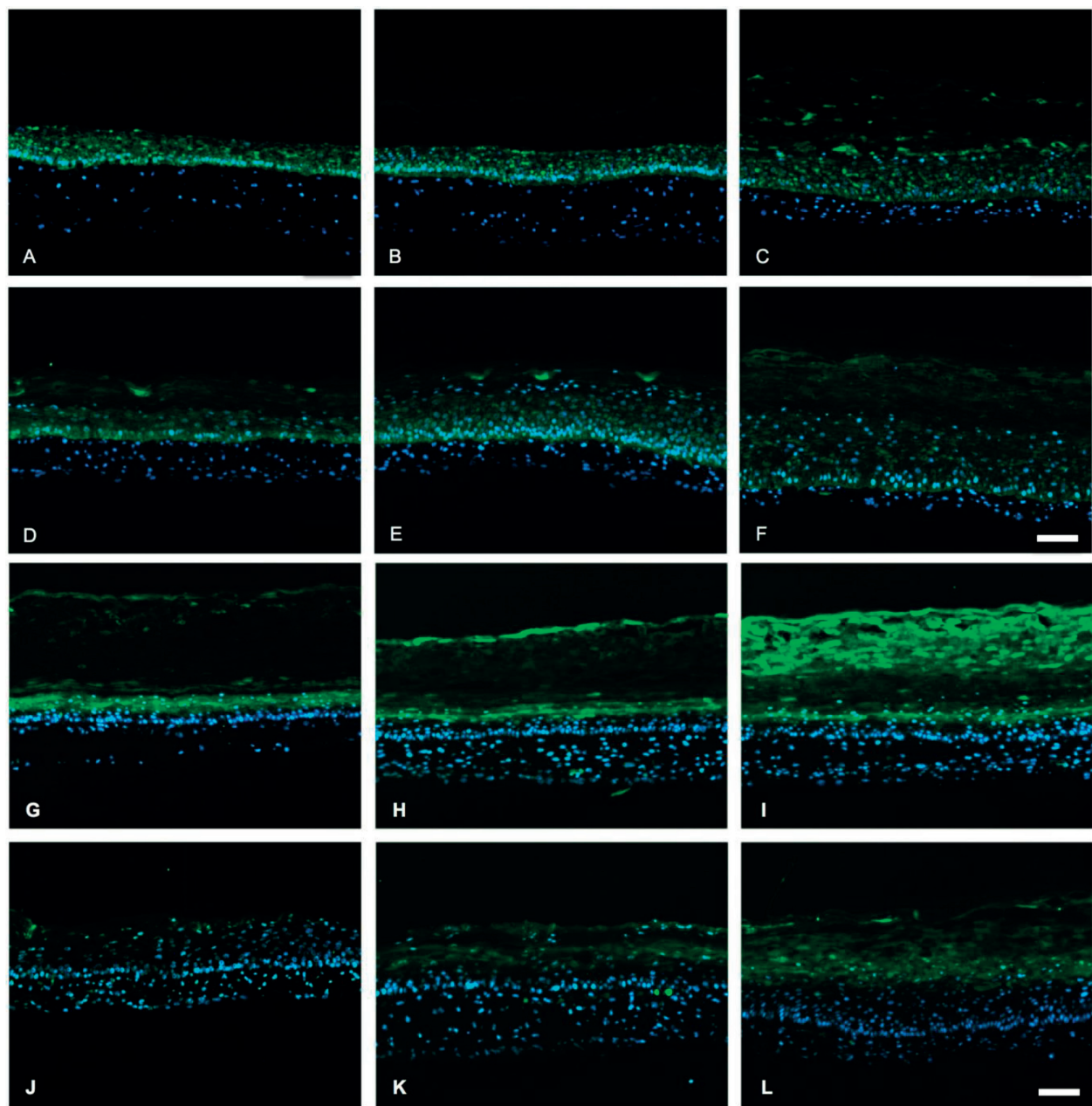
## 2.2. Discussion

With psoriasis etiology still unknown, several *in vivo* models of psoriasis have been generated for a better understanding of the pathology. Genetically modified, spontaneous mutations, cytokine injections, xenografts, and gene knockout mouse models are all examples [21, 39–41]. Although these models showed psoriasis-like lesions, psoriasis is a specific human disease, and mouse skin does not represent characteristics of human skin such as epidermal thickness and structure, rete ridges and differences in certain immune cells [40, 41]. Due to the advances that have been made in the field of tissue engineering in the past years, it has been possible to develop *in vitro* psoriatic skin models to understand the disease, improve the development of new treatments, and limit the use of animals [24, 25, 28, 42]. Our team has developed a representative psoriatic skin model reconstructed according to a self-assembly method showing *in vivo* features of psoriasis such as hyperproliferation and abnormal differentiation of



**Figure 5.** Filaggrin and loricrin staining. Expression of filaggrin (red) in healthy substitutes (A–C), psoriatic substitutes (D–F) and substitutes produced according to the original method (A and D), the 6-well plate modification (B and E), and the 12-well plate modification (C and F). Expression of loricrin (green) in healthy substitutes (G–I), psoriatic substitutes (J–L) and substitutes produced according to the original method (G and J), the 6-well plate modification (H and K), and the 12-well plate modification (I and L). The nuclei were stained with Hoechst (blue). Three substitutes of each condition were analyzed, and the results were confirmed with three independent experiments. Cells from two different healthy patients and three different psoriatic patients were used (scale bar = 100  $\mu$ m).

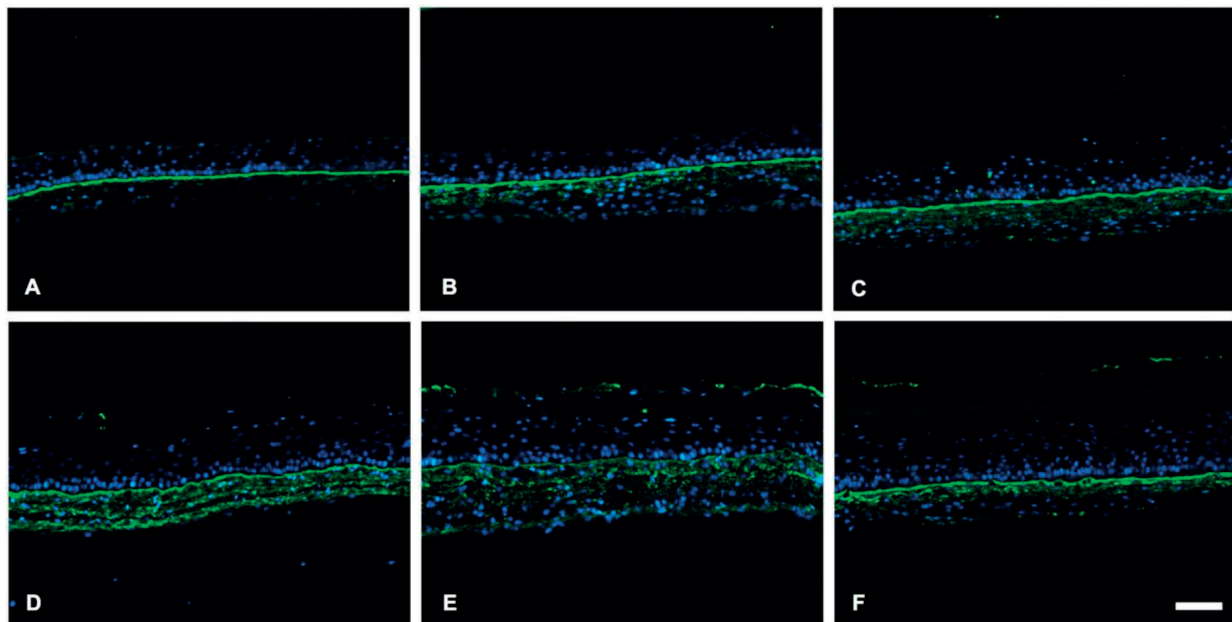
keratinocytes [31]. However, each model has limitations, and some of the model developed in our team is the large number of cells required for the production of skin substitutes, in addition to complex manipulations that may generate less reproducibility. Therefore, the optimization of the production of psoriatic skin substitutes with the aim of making them suitable models for their use in pharmacological testing remains a challenge.



**Figure 6.** Keratin 14 and keratin 1 staining. Expression of keratin 14 (green) in healthy substitutes (A–C), psoriatic substitutes (D–F) and substitutes produced with the original method (A and D), the 6-well plate modification (B and E), and the 12-well plate modification (C and F). Expression of keratin 1 (green) in healthy substitutes (G–I), psoriatic substitutes (J–L) and substitutes produced according to the original method (G and J), the 6-well plate modification (H and K), and the 12-well plate modification (I and L). The nuclei were stained with Hoechst (blue). Three substitutes of each condition were analyzed, and the results were confirmed with three independent experiments. Cells from two different healthy patients and three different psoriatic patients were used (scale bar = 100  $\mu$ m).

Previous studies of our group have shown that this psoriatic skin model demonstrated phenotypic characteristics of *in vivo* psoriasis [31–33]. In the present study, it is demonstrated that the improvements made to the original self-assembly method did not affect the psoriatic phenotype of the substitutes. *In vivo* psoriatic features are still expressed in the substitutes reconstructed according to the 6-well plate and 12-well plate modifications to protocol. Indeed,





**Figure 7.** Laminin staining. Expression of laminin (green) in healthy substitutes (A–C), psoriatic substitutes (D–F) and substitutes produced according to the original method (A and D), the 6-well plate modification (B and E), and the 12-well plate modification (C and F). The nuclei were stained with Hoechst (blue). Three substitutes of each condition were analyzed, and the results were confirmed with three independent experiments. Cells from two different healthy patients and three different psoriatic patients were used (scale bar = 100  $\mu$ m).

in both new protocols, a significant increase in the living epidermis thickness of psoriatic substitutes is observed compared with their respective healthy substitute counterparts as for the original method. Interestingly, the epidermal thickness of psoriatic substitutes produced according to the 12-well plate modification was significantly thicker than those produced according to the original method. This difference could be explained by the same seeding number of keratinocytes in a smaller seeding area for the 12-well plate protocol. Thus, this induces an increase of acanthosis. Considering histological analysis, our model stands out from other models of *in vitro* psoriatic skin that did not show acanthosis [24, 25]. However, based on these histological observations, the 6-well plate modification to protocol seems to be the most representative compared with the original protocol.

Differentiation of psoriatic skin is characterized by the altered expression of several epidermal proteins [43]. In a normal differentiation process (approximately 28 days), the basal layer transit amplifying cells differentiate and migrate into upper epidermal layers and synthesize important proteins involved in the differentiation and the skin barrier function such as filaggrin, loricrin, and keratins [36, 37, 44]. Filaggrin is normally synthesized from a precursor, profilaggrin, found in the granular layer. This protein is a key role in the formation of the cornified envelope [37]. In skin disease, such as psoriasis, expression of filaggrin is decreased, such that it is sometimes even absent due to an altered differentiation process and a reduction or an absence of the granular layer (agranulose) [38, 45, 46]. Loricrin, a major component of the cornified envelope, is stored in granules of the *stratum granulosum*, and its expression is also decreased or absent in psoriatic skin [36, 47]. In agreements with these observations, these features were observed with the substitutes reconstructed according to the original method



and the two modifications to protocol (6-well plate and 12-well plate modifications). Indeed, filaggrin and loricrin were detected in healthy skin substitutes, whereas their absence was observed in psoriatic skin substitutes. This therefore confirms that the characteristics associated with the psoriatic phenotype are preserved with these new methods.

Keratins are intermediate filaments highly involved in epidermal structure and different types are expressed in the varying differentiation stages [48]. K5 and K14 are normally found in the basal layer of the epidermis, and they are progressively replaced by K1 and K10 in suprabasal layers [49]. However, *in vivo* psoriatic skin shows K14 in all layers of the epidermis, including the *stratum corneum*. This therefore suggests that the degradation mechanism of this keratin is altered in psoriasis [48, 50]. Moreover, in such hyperproliferative diseases, a new pair of keratins, K6 and K16, is appearing, causing a decrease in the expression of K1 and K10 [48, 50–52]. In the present work, healthy and psoriatic skin substitutes reconstructed according to the original method and the 6-well plate modification to protocol have demonstrated the same K1 and K14 expression than *in vivo*. Thus, taking together, K1, K14, filaggrin, and loricrin results validated the conservation of psoriatic skin differentiation in the new 6-well plate modification. This suggests that the 6-well method would be a great alternative to the original method. In healthy substitutes produced following the 12-well plate modification, an abnormal presence of K14 and K1 was observed in the *stratum corneum*, showing a less efficient differentiation of keratinocytes. This can probably be explained by the higher number of cells seeded in the culture area. These observations demonstrated that the 6-well plate modification is more effective for the production of healthy substitutes than the 12-well plate modification.

Some studies suggested that alterations in the basal membrane of psoriatic skin play an important role in the abnormal proliferation and differentiation of psoriatic keratinocytes [53–55]. Indeed, the expression of proteins such as laminin, which is one of the main proteins that forms the basal membrane, is decreased and disrupted in psoriatic skin unlike in healthy skin. In this last, laminin forms a linear and continuous structure [54]. Laminin expression in our skin substitutes produced with healthy cells regardless of the method (original method and two new modifications) was intense, continuous and more restricted to the basal lamina, demonstrating a good structure of the basal membrane. For psoriatic skin substitutes (original method and 6-well plate modification), the expression of laminin was more distributed through the dermis compared to healthy substitutes, showing disorganization in the basal membrane. For the psoriatic skin substitutes reconstructed according to the 12-well plate modification, laminin staining was more compact and similar to healthy substitute expression. Interestingly, this observation is showing that the basal membrane was more organized, thus less similar to the psoriatic phenotype. These results showed that the 6-well plate modification is more representative of the *in vivo* psoriatic skin than the 12-well plate modification.

### 2.3. Conclusion

These new modifications to protocol provide several advantages in the production of skin substitutes. Indeed, the 6-well and 12-well plate modifications require almost 3 times fewer

fibroblasts and culture medium than the original method for the production of fibroblast sheets, which favorably reduce production costs. In addition, the use of plates and anchoring papers at the beginning of the production greatly facilitate the handling and the superposition of fibroblast sheets increasing the quality of the dermal equivalent. The anchoring papers allow the production of more reproducible and uniform size substitutes within the same experiment and between the different studies, which are essential for pharmaceutical studies. In conclusion, the modifications made to the original self-assembly method for the production of psoriatic substitutes are effective and demonstrate a comparable phenotype. However, the 6-well method is the one that leads to reconstructed substitutes with characteristics more similar to those seen *in vivo*. These modifications make it possible to obtain substitutes that are distinguished by better reproducibility making them new tools of choice for pharmacological analyses.

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## Conflict of interest

The authors state no conflict of interest. The authors have no relationship with a for-profit or a not-for-profit organization to disclose. There is no financial conflict with the subject or the materials discussed in the manuscript apart from those disclosed.

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